DEFERRALS

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 11-19-74

1. Principal Investigator (give title and degrees):

Carl G. Becker, M.D., Assoc. Professor of Pathology, Principal Investigator

Gregory Siskind, M.D., Assoc. Professor of Medicine, Consultant in Immunology

2. Institution & address:

Cornell University Medical College
Department of Pathology
1300 York Avenue
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Investigation of the Role of Allergy to Tobacco Constituents in the Pathogenesis of Arteriosclerosis and Myocardial Infarction.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Smoking of tobacco and atherosclerosis have been associated statistically (1-4). The pathogenetic mechanisms that are the basis of this relationship are obscure. Research proposed herein is designed to examine the hypothesis that certain people become allergic to constituents of tobacco or tobacco smoke and that injury to blood vessels, arteriosclerosis, and injury to the myocardium may be consequent to repeated challenge with tobacco allergens (5-7). Scarring of pulmonary blood vessels has been observed with greater frequency in lungs of cigarette smokers than in lungs of pipe or cigar smokers or non-smokers (8).

In the course of these investigations we propose to:

a) Isolate, purify and identify as allergenic, material from tobacco. We propose to identify the route of entry and absorption of this allergenic material.

b) Examine sera from non-smokers and smokers with and without clinical evidence of ischemic heart disease and with and without pulmonary emphysema for the presence of reaginic (IgE) antibodies

Research Aims (cont'd)

to tobacco antigens by radioimmunoassay technique. We propose to skin test the same subjects for immediate and delayed hypersensitivity to these antigens. Because of recent data indicating that in rabbits production of anaphylactic or IgE antibodies can be especially stimulated by neonatal immunization, we propose to examine the sera of children from smoking and non-smoking mothers for IgE antibodies to tobacco antigens. If possible, we shall also skin test these children.

8. Brief Statement of Working Hypothesis:

The hypothesis underlying the proposed research is that some tobacco smokers become sensitized to one or more allergenic components of tobacco smoke and that repeated challenge with these allergens by smoking can result in immunologic injury to blood vessels, accentuating the development of athero-arteriosclerosis and increasing the risk of myocardial infarction. Similarly, repeated immunologic injury to pulmonary blood vessels may contribute to the development of pulmonary emphysema. A related, additional hypothesis is that some smokers sensitized to tobacco allergens can develop myocardial anaphylaxis on repeated contact with these allergens.

9. Details of Experimental Design:

A. Purification and Identification of Tobacco Allergens:

1) Tobacco Leaf (Glycoprotein (TGP) --

We have extracted cured tobacco leaves of Maryland, Turkish, Burley, and Flue cured varieties (obtained through the courtesy of American Tobacco Co.) with phosphate buffered saline, pH 7.4 (PBS) after delipidization with petroleum ether. The saline extracts were brought to 60% saturation with crystalline $(NH_4)_2SO_4$, redissolved in PBS and applied to G-25 Sephadex columns from which it emerged with the void volume as a single, sharp, symmetrical peak. When this peak was applied to 7.5% polyacrylamide gels, pH 8.9 and subjected to electrophoresis it migrated rapidly and anodally as a single, brown band which stained with both protein stains (Amido black or Coomassie blue) and the PAS reaction, indicating that it is a glycoprotein (Fig. 1).

The electrophoretic migration and staining characteristics of material extracted from the four different kinds of tobacco were identical (Fig. 2). This purified material, when injected intradermally induced wheal and flare reactions within 30 minutes of intradermal injection in three of six smokers and in one non-smoker with a history of rhinorrhea in response to tobacco

Details of Experimental Design (cont'd)

smoke exposure. It produced no reaction in two of two non-smokers. This material is presumably the allergenic principle present in tobacco extracts described by Harkavy and Perlman (5). For purposes outlined in this research proposal tobacco leaf glycoprotein antigen (TGP) will be prepared as outlined above.

2) Tobacco Leaf Lipids --

a) Rhus toxin is volatilized when leaves of the genus Rhus (poison ivy, poison oak, poison sumac) are burned, yet still capable of inducing contact dermatitis by mechanisms related to delayed hypersensitivity in sensitive subjects. We propose to examine the lipid soluble material from cured tobacco leaves for allergenic material that on burning become volatilizable haptens.

b) The petroleum ether extract of cured tobacco leaves that is obtained from the first stage of preparation of TGP will be concentrated by evaporation of the petroleum ether. The resulting material will be assayed for allergenicity as described below.

B. Demonstration and Characterization of Antibodies to Tobacco Leaf Glycoprotein (TGP) in Human Serum:

1) The purpose of this phase of the proposed research is to determine if smokers develop humoral antibodies to TGP, to determine the frequency of immunization, and to determine which class of antibodies are formed.

2) Characterization and quantitation of the antibodies to TGP.

a) TGP will be coupled to Sepharose beads activated with cyanogen bromide to prepare an insoluble immunoabsorbent (S-TGP) (9).

b) Aliquots of serum from subjects described above will be incubated with S-TGP and then centrifuged and washed several times.

c) After step (b), aliquots of S-TGP will then be incubated with I^{125}-labeled antibodies specific for heavy chains of IgE, IgG, IgA and IgM. After centrifugation and washing, the radioactivity will be counted and the quantity of immunoglobulin specifically bound to S-TGP calculated from previously prepared standard curves.

d) If it is demonstrated that some smokers have significantly elevated levels of IgE antibodies specifically directed against TGP and other smokers or non-smokers have no IgE anti-TGP we propose to test volunteers from both groups for immediate hypersensitivity to TGP by intradermal injection of TGP in doses of 10 and 50 µgrams. This will serve as a bioassay control for the radioimmunoassay of IgE antibodies specific for TGP.

Details of Experimental Design (cont'd)

3) The radioimmunoassay procedure described above will be used to examine serum samples obtained from the following groups of people for antibodies to TGP:

a) smokers without evidence of ischemic heart disease,

b) age-matched smokers with evidence of ischemic heart disease,

c) age-matched non-smokers,

d) patients with thromboangiitis obliterans,

e) smokers with clinical evidence of emphysema

f) non-smokers with clinical evidence of emphysema or other chronic pulmonary disease,

g) smoking and non-smoking medical students,

h) former smokers who have given up smoking.

We would begin by studying blood samples from our medical student class from whom we will also obtain a history of smoking habits and of allergies. These serum samples will be used to establish the assay technique as well as to obtain data on our student population. Once the radioimmunoassay procedures are found to be satisfactory we will study serum samples from the other patient groups. In this connection I have been promised the help of our Division of Cardiology in obtaining serum samples from patients with ischemic heart disease or peripheral vascular disease. Similarly, the physicians staffing our pulmonary clinic have also expressed great interest in this project and have promised cooperation in obtaining serum samples from patients with chronic pulmonary disease. Finally, Dr. Lawrence Hinkle, Director of the Cornell Human Ecology Program, studying events contributing to myocardial infarction and sudden death in employees of the Telephone Company has agreed to provide us with serum specimens from human subjects in this study. Extensive data on smoking and dietary habits as well as physical examinations, measurements of serum cholesterol, etc, have been collected on these patients. We will have an opportunity to compare smokers, non-smokers, and former smokers with respect to antibodies to tobacco antigens. These patients have also been evaluated for presence or absence of clinical evidence of arteriosclerotic cardiovascular disease. If we can demonstrate that some smokers are indeed immunized to tobacco products, we can then compare a population of smokers with and without clinical evidence of arteriosclerotic cardiovascular disease with respect to the presence or absence of antibodies to tobacco constituents.

4) Characterization of the route of absorption of TGP.

a) The question arises as to how TGP is absorbed. Is a hapten released from it when tobacco is burned? Or,

Details of Experimental Design (cont'd)

is TGP taken in with steam generated behind burning tobacco? In this connection, an allergenic muscle protein has been demonstrated in the steam from cooking codfish (10). We propose to examine saliva collected from smoking subjects and condensates of tobacco smoke for the presence of intact TGP or fragments of TGP present as haptens. We also propose to examine the blood of smokers for TGP antigen. A radioimmunoassay using I^{125}-labeled TGP and competitive protein binding technique will be the means of these measurements.

b) Antisera to TGP will be prepared in rabbits. Antibody to TGP will be purified from sera from immunized rabbits using columns of TGP coupled to Sepharose.

c) TGP will be radio-labeled and the capacity of standard anti-TGP sera to bind TGP determined. Antibody will then be incubated with known amounts of cold TGP and then with labeled TGP and a standard curve constructed.

d) Saliva and blood of subjects will be collected before, during, and after smoking. We will look for TGP on saliva and serum samples by measuring the capacity of the samples to inhibit binding of radiolabeled TGP to anti-TGP.

e) If there is evidence of TGP in saliva, a large quantity of saliva will be collected from smoking subjects. In order to see if TGP is present in intact form we shall attempt to reisolate it from saliva and compare it physically and immunochemically with TGP isolated from tobacco leaves.

f) Failure to reisolate intact TGP from saliva containing TGP reactive with anti-TGP would suggest that it is present as a hapten. If so, we shall attempt to isolate the hapten by using anti-TGP coupled to an insoluble matrix.

g) We also propose to examine condensates of tobacco smoke for TGP by radioimmunoassay as well as attempt to isolate intact and/or haptenic TGP from these condensates.

h) It is conceivable that other of the family Solanaceae, i.e. potatoes, tomatoes, green pepper, eggplant, have antigens similar to tobacco and that some people become sensitized consequent to ingesting these antigens. When they smoke, they challenge themselve with similar or cross reactive antigens. To test this hypothesis we propose to extract potatoes, tomatoes, green peppers,

Details of Experimental Design (cont'd)

and eggplant to see if we can demonstrate antigen similar to TGP. Glycoprotein extracted from these sources will be compared with TGP by double diffusion in agarose gel against rabbit antisera to highly purified TGP. If in the course of experiments described herein, other antigens are obtained from tobacco to which smokers are allergic, we shall see if these antigens are also distributed among the other Solanaceae.

C. Assay of Tobacco Leaf Lipid (TLL) for Allergenic Activity:

1) It is not known whether any of the lipid soluble material in tobacco leaf is allergenic. In fact, in two recent publications dealing with the chemical nature of allergens, there is no mention of allergenic lipids (11,12).

2) The crude lipid extract will be prepared as described above. Since this is a complex mixture of unknown properties we will assay it for allergenicity by indirect means rather than by skin testing human subjects.

3) Some subjects allergic to tobacco glycoprotein (TGP) might well be allergic to TLL as well. Serum from subjects with high levels of IgE antibodies to TGP and serum from subjects with no antibodies to TGP will be injected intradermally into a Rhesus monkey. After 50 hours and immediately following systemic injection of Coomassie blue, the sites of serum injection will be injected with TLL (Prausnitz-Kustner (P-K) reaction) and observed for blueing. Alternatively, the TLL will be injected intravenously and the sites of serum injection observed for blueing (Passive Cutaneous Anaphylaxis (PCA) reaction). The TLL may not be soluble in saline, in which case we would extract TLL with the monkey's own serum in the hope that some of the lipid components might bind to serum albumin and/or lipoproteins. The TLL serum extract would then be used for P-K or PCA reactions.

4) Blood will be drawn from subjects with and without reaginic antibodies to TGP and the capacity of TLL to stimulate histamine release from their leukocytes measured (13).

5) If the lipid extracts are shown to have allergenic activity, we propose to fractionate the lipid extract to isolate the specific allergen(s) using techniques of thin layer chromatography on silica gels, solvent resistant Sephadex, etc.

6) Lipid fractions obtained in paragraph (5) would be assayed as in paragraphs (3) and (4) for allergenic activity.

Details of Experimental Design (cont'd)

7) Purified lipid fractions demonstrated to have allergenic activity will be coupled to insoluble matrices and human sera will be examined for antibodies to them by radioimmunoassay technique as described above for TGP. If the lipid fractions are nontoxic we would also attempt skin testing of human subjects.

8) If it can be shown that certain lipid components of tobacco leaf are allergenic we would like to examine condensates of tobacco smoke for their presence, employing techniques of thin layer chromatography or gas liquid chromatography. Lipids recovered from tobacco smoke and corresponding chromatographically to those isolated from tobacco leaf will be assayed for allergenicity as described above.

References

1. Auerbach O, Hammond ED and Garfinkel L: Smoking relation to atherosclerosis of the coronary arteries. NEJM 273:2686, 1965.

2. Strong JP, et al: On the association of cigarette smoking with coronary and aortic atherosclerosis. J Athero Res 10: 303, 1969.

3. Sackett DL, et al: Relation between aortic atherosclerosis and the use of cigarette and alcohol. NEJM 279:1413, 1968.

4. Hatch FT, et al: A study of coronary artery disease in young men: Characteristics and metabolic studies of the patients and comparison with age-matched young men. Circ 33:679, 1966.

5. Harkavy J and Perlman E: Tobacco allergy in coronary artery disease. NY State J of Med 64:1287, 1964.

6. Harkavy J: Cardiac manifestations due to allergy. NY State J of Med 64:2289, 1964.

7. Harkavy J: Cardiovascular manifestations due to hypersensitivity. NY State of Med 69:2757, 1969.

8. Auerbach O, et al: Smoking habits and age in relation to pulmonary changes: rupture of alveolar septums, fibrosis, and thickening of walls of small arteries and arterioles. NEJM 269:1045, 1963.

9. Axen R, Porath J, and Sverker E: Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature 214:1302, 1967.

Details of Experimental Design (cont'd)

10. Aas K and Jebsen JW: Studies of hypersensitivity to fish: Partial purification and crystallization of a major allergenic component of cod. Int Arch Allergy 32:1, 1967.

11. Berrens L: The chemistry of atopic allergens. Monographs in Allergy, vol 7. S Karger, New York, 1971.

12. Stanworth DR: Immediate hypersensitivity. The molecular basis of the allergic response. American Elsevier Publishing Co, New York, 1973.

13. May CD: Procedures for immunochemical study of histamine release from leukocytes with small volumes of blood. J Allergy 46:12, 1970.

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10. Space and Facilities Available:

1) Laboratory Space --

Four large laboratories with hoods for analytical chemistry, lipid chemistry, histochemistry, and processing tissues for electron microscopy.
One small laboratory for bacteriology.
One small laboratory for immunochemistry and protein chemistry.
One small laboratory for ultramicrotomy for electron microscopy.
One small utility room for inoculations, bleeding of rabbits, preparation of special diets, and autopsy of animals.
One utility room with autoclave, automatic glassware washer, drying oven, and distillation apparatus.
Two 4°C cold rooms for protein chemistry and storage of special diets.
One room for tissue culture. This room is equipped with a small cubicle for sterile transfer, a CO_2 incubator, and an inverted microscope.

2) Offices for each investigator and a technician/secretary.

3) Animal Quarters --

One large animal room with 40 breeding cages and 150 other cages for maintenance of specially bred stock of rabbits and other experiments.
One small room for isolation and maintenance of approximately 50 rabbits receiving streptococcal infections. In addition, this room has 15 metabolism cages for collection of urine from infected rabbits.

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Space and Facilities Available (cont'd)

One small animal room with cages for 80 rabbits fed lipid-rich diets.
One small animal room with cages for approximately 40-50 rabbits with foreign serum induced hypertension. In addition, this room contains 9 metabolism cages for collection of urine from hypertensive and nonhypertensive rabbits. (It also contains cages for approximately 10 guinea pigs for immunization).
One small room for storage of food, shavings, and cleaning equipment for the animal rooms.
One small room with cage cleaning facilities.
Three refrigerators - 2 for storage of dead animals and 1 for storage of special diets.

4) One Siemens Elmiskop I and one RCA EMU 3-B electron microscope with two dark rooms, one for developing plates and one for printing (departmental facility shared with other investigators).

5) A scanning electron microscope, ETEC Ultrascan, has recently been acquired as an institutional instrument and is readily available for the use of Dr. Minick and Dr. Insull one day per week. In addition, there is considerable evening and weekend time available. The facility includes a dark room, a critical point dryer, vacuum evaporator and stereoviewer. A cryostage and micromanipulator are to be delivered.

6) Facilities for ultramicrotomy, including one Porter Blum MT-1 ultramicrotome, one LKB Ultratome with special cutting table, one LKB knife maker, one dissecting microscope, and an oven for polymerization of embedding materials.

7) Histology laboratory facilities, including 2 American Optical microtomes, one knife sharpener, staining equipment, and paraffin oven. A Technicon is shared with the departmental histology laboratory.

8) One Beckman DB spectrophotometer equipped with automatic wavelength programmer and potentiometric recorder and 1 Coleman junior spectrophotometer.

9) One refrigerated high speed centrifuge, Beckman LB75 preparative ultracentrifuge, and 3 table centrifuges.

10) Electrophoresis equipment. Buchler apparatus for agar gel immunoelectrophoresis, analytical polyacrylamide electrophoresis and preparative polyacrylamide electrophoresis. Equipment for starch gel, starch block and agarose block electrophoresis.

Space and Facilities Available (cont'd)

11) Equipment for column chromatography including 2 fraction collectors (one fraction collector is very worn and must be replaced).

12) One bacteriology incubator, 3 water baths, and 1 constant temperature bath. Two ovens for drying and evaporation.

13) One Braun disintegrator for grinding bacteria.

14) Two distilling apparatuses, one for distillation of lipid solvents and one for double distilling water.

15) Three deep freezers and four refrigerators.

16) One vacuum-freeze lyophilizer.

17) One IEC cryostat. (The latter is departmental equipment shared with others).

18) One analytical balance, three torsion balances, one beam balance and two scales for weighing rabbits and feed. The torsion balance in the animal room is over 10 years old and badly worn and must be replaced.

19) One Grass model 5P1 polygraph with four channels. One strain gauge pressure transducer. (This equipment is on loan from another department, must be returned, and a replacement purchased.)

20) Two pH meters.

21) Four microscopes and 2 photomicroscopes (both adapted for fluorescence microscopy).

22) Photographic apparatus for photographing gross pathology specimens.

23) One Nuclear Chicago Gamma Radiation Counter, 1185 series.

24) One Nuclear Chicago Liquid Scintillation Counter, Isocap 300.

11. Additional Facilities Required:

None

12. Biographical Sketches of Investigators:

Curricula of Dr. Becker and Dr. Siskind are appended.

13. Pertinent Publications:

Reprints of the 15 publications listed below that are pertinent to this application have been supplied earlier (July 15th, 1974) in triplicate to the offices of The Council for Tobacco Research.

1) Minick CR, Murphy GE and Campbell WG Jr: Experimental Induction of Athero-arteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J Exp Med, 124, no 4, 635-652, 1966.

2) Minick CR and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich, cholesterol-poor diet. Am J Path, 73, no 2, 265-300, 1973.

3) Hardin NJ, Minick CR, and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. III. The role of earlier acquired fibromuscular intimal thickening in the pathogenesis of later developing atherosclerosis. Am J Path, 73, no 2, 301-326, 1973.

4) Becker CG and Murphy GE: Demonstration of Contractile Protein in Endothelium and Cells of the Heart Valves, Endocardium, Intima, Arteriosclerotic Plaques, and Aschoff Bodies of Rheumatic Heart Disease. Am J Path, 55, no 1, 1-22, 1973.

5) Becker CG and Nachman RL: Contractile Proteins of Endothelial Cells, Platelets and Smooth Muscle. Am J Path, 71, no 1, 1-22, 1973.

6) Becker CG: Demonstration of Actomyosin in Mesangial Cells of the Renal Glomerulus. Am J Path, 66, no 1, 97-110, 1972.

7) Jaffe EA, Nachman RL, Becker CG and Minick CR: Culture of Human Endothelial Cells Derived from Umbilical Veins. Identification of morphologic and immunologic criteria. J Clin Invest, 52, no 11, 2745-2756, 1973.

8) Levi R: Hypersensitivity Reactions of the Heart: An Experimental Model. Bul NY Acad Med, 46, no 11, 997, 1970.

9) Levi R: Effects of Exogenous and Immunologically Released Histamine on the Isolated Heart: A Quantitative Comparison. J Pharm and Exp Therapeut, 182, no 2, 227-238, 1972.

Pertinent Publications (cont'd)

10. Capurro N and Levi R: Anaphylaxis in the Guinea-Pig Isolated Heart: Selective inhibition by burimamide of the positive inotropic and chronotropic effects of released histamine. Brit J Pharm, 48, no 4, 620-628, 1973.

11. Levi R and Capurro N: Histamine H_2-Receptor Antagonism and Cardiac Anaphylaxis. Proc Intl Symp on Histamine H_2-Receptor Antagonists, Eds CJ Wood and MA Simkins, Smith Kline and French, London, 175-183, 1973.

12. Capurro N and Levi R: The Heart as a Target Organ in Systemic Allergic Reactions: Comparison of Cardiac Anaphylaxis in vivo and in vitro. Submitted for publication to Circulation Research, July 1974.

13. Harkavy J and Perlman E: Tobacco Allergy in Coronary Artery Disease. NY State J of Med, 64, no 11, 1287-1296, 1964.

14. Harkavy J: Cardiac Manifestations Due to Allergy. NY State J of Med, 64, no 18, 2291-2302, 1964.

15. Harkavy J: Cardiovascular Manifestations Due to Sensitivity. NY State J of Med, 69, no 21, 2757-2765, 1969.

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount	Fringe
Professional (give % time of investigator(s) even if no salary requested)			
None			
Technical			
1. Immunochemistry technician	80%	$10,000	$1,575
2. General laboratory and animal technician	70	6,666	1,166
Sub-Total for A		$19,407	

B. Consumable supplies (by major categories)

1. Rabbits in which to make anti-TGP antibody. Two goats for preparation of antibody to human IgE. Approximately 6 Rhesus or African green monkeys for passive sensitization experiments with human sera (Prausnitz-Kustner reactions)	1,400
2. Chemicals, biologicals, isotopes, glassware, disposable test tubes, pipettes, etc. for radioisotope work, stationery	4,000
Sub-Total for B	$5,400

C. Other Expenses (itemize)

Maintenance and service contract costs on centrifuges, counting equipment, etc.	
Sub-Total for C	$1,200
Running Total of A + B + C	$26,007

D. Permanent equipment (itemize)

None

Sub-Total for D	
E. Indirect costs (15% of A+B+C) E	3,901
Total request	$29,908

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Contractile Proteins of Vascular Smooth Muscle*	Am. Heart Assoc. #72-900	$20,365	7/1/74 to 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount/yr.	Inclusive Dates
Arteriosclerosis, glomerulonephritis and rheumatic fever*	NIH HL-01803	$150,000	7/1/75 to 6/30/80 if approved and funded
Pathogenesis of Post-streptococcal Glomerulonephritis	Am. Heart Assoc.	26,290	7/1/75 to 6/30/78
Contractile and Regulatory Proteins of Blood Vessels and Platelets	N.Y. Heart Assoc.	20,011	7/1/75 to 6/30/77

*Research on tobacco is not proposed in any of these grants.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carl G. Becker, M.D.

Signature [signature] Date 11/26/74

Telephone (212) 472-5984
Area Code Number Extension

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Cornell University Medical College

Mailing address for checks
1300 York Avenue
New York, New York 10021

Responsible officer of institution

Typed Name _____

Title _____

Signature [signature] Date 12-2-74

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